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<b>13. ABSTRACT (Maximum 200 Words)</b> While bony metastases of prostate cancer are often osteoblastic, excessive bone resorption also occurs, which contributes to skeletal complications (e.g., pain, fractures). This research evaluates whether prostate cancer cells express the extracellular calcium (Ca <sup>2+</sup> )-sensing receptor (CaSR) and whether the CaSR in bony metastases of prostate cancer participates in a vicious cycle involving CaSR-mediated secretion of the bone-resorbing cytokine, parathyroid hormone-related protein (PTHrP). The secreted PTHrP would promote further bone resorption, thereby increasing Ca <sup>2+</sup> locally and stimulating further PTHrP release. The project entails four tasks--namely showing that: (1) prostate cancer cells express the CaSR, (2) the CaSR mediates high Ca <sup>2+</sup> -induced stimulation of PTHrP secretion, (3) the CaSR transactivates the EGF receptor, and (4) CaSR-stimulated PTHrP secretion from prostate cancer cells increases the severity of metastatic bone disease in vivo in mice. We have accomplished tasks 1 and 2, shown that the CaSR transactivates the CaSR in task 3 and are in the process of developing the stably transfected cell lines needed for the studies in task 4. These results support a role for the CaSR in a vicious cycle that increases the severity of bone resorption in vivo in humans.				
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## INTRODUCTION:

Prostate cancer research has generally emphasized the osteoblastic nature of prostate cancer metastases to bone. However, a wealth of recent data documents the nearly universal presence of excessive bone resorption as well, which participates importantly in the associated bone pain and fractures. The goal of this research is to determine whether prostate cancer cells express the extracellular calcium ( $\text{Ca}^{2+}_o$ )-sensing receptor (CaSR) and whether the CaSR participates in a vicious cycle promoting excessive bone resorption. This vicious cycle involves CaSR-mediated secretion of the bone-resorbing cytokine, parathyroid hormone-related protein (PTHrP), by prostate cancer metastatic to bone. The secreted PTHrP would produce further bone resorption, which would elevate the local level of  $\text{Ca}^{2+}_o$ , thereby stimulating further PTHrP release by the prostate cancer cells, and so forth. The scope of the project encompasses four specific aims: (1) to show that prostate cancer cells express the CaSR; (2) to prove that the CaSR mediates high  $\text{Ca}^{2+}_o$ -evoked stimulation of PTHrP secretion *in vitro*; (3) to determine whether the CaSR initiates a paracrine pathway producing transactivation of the EGF receptor, which then produces EGFR-mediated stimulation of MAPK and, in turn, increased PTHrP production; and (4) to document that CaSR-mediated stimulation of PTHrP secretion from prostate cancer cells injected into the femora of nude mice contributes to the severity of metastatic bone disease by knocking out the receptor using a dominant negative CaSR construct. The progress that has been made in the tasks related to these specific aims relative to the time frames originally proposed for those tasks is detailed below.

## BODY:

*Task 1—To document that prostate cancer cell lines express the CaSR (months 1-18).*

We have completed the studies in task 1, which are described in detail in a publication of this work submitted with the previous Annual Report (1). The results of these studies are as follows: Reverse transcriptase-polymerase chain reaction (RT-PCR) with intron-spanning primers amplified a product of the expected size, 480 bp, for having been derived from authentic CaSR transcript(s). In addition, Northern analysis, carried out using a CaSR-derived riboprobe and poly(A<sup>+</sup>) RNA derived from both LnCaP and PC-3 cells, revealed a major transcript of 5.2 kb, which is of the same size as the major transcript in human parathyroid gland (2).

With regard to documenting the presence of CaSR protein, immunocytochemistry with a polyclonal, CaSR-specific antiserum revealed specific staining of both PC-3 and LnCaP cells. Furthermore, western blotting with the same antiserum identified specific immunoreactive bands of 160-170 kDa in PC-3 and LnCaP cells, comparable in size to bands identified in the positive

controls—bovine parathyroid gland and CaSR-transfected human embryonic kidney (HEK293) cells (1).

Thus we have demonstrated that LnCaP as well as PC-3 cells express both CaSR transcript and protein. Note that while we originally proposed studies determining whether prostate cancer specimens removed at the time of prostatectomy expressed CaSR transcript(s) and protein, the contract for our grant expressly forbids the use of human anatomical substances. Should the contract be amended, however, we would be pleased to carry the latter studies out during months 24-36.

*Task 2—To show that the CaSR mediates the stimulation of PTHrP secretion from prostate cancer cell lines by high  $\text{Ca}^{2+}$ . (months 6-24).*

To investigate whether the CaSR mediates the stimulatory effect of high  $\text{Ca}^{2+}$  on PTHrP secretion from PC-3 cells (1), we utilized polycationic agonists (i.e., neomycin and spermine) known to activate the cloned CaSR (3, 4). These two polycations were equal to or more effective than high  $\text{Ca}^{2+}$  in stimulating PTHrP secretion from PC-3 cells. We next used a naturally occurring, dominant negative construct of the CaSR (R185Q) to assess the CaSR's role in mediating high  $\text{Ca}^{2+}$ -evoked PTHrP secretion. To achieve high efficiency expression of the CaSR, we utilized infection with an adenoviral construct. Compared to vector-infected cells, cells infected with the dominant negative CaSR showed a substantial reduction in the stimulation of PTHrP secretion by 1.5 and 3.5 mM  $\text{Ca}^{2+}$  (1), levels of  $\text{Ca}^{2+}$  that could potentially be encountered by bony metastases of prostate cancer near sites of active bone resorption (5).

*Task 3—To investigate whether the CaSR transactivates the EGFR in prostate cancer cells (months 6-24).*

In addition to the studies accomplished in tasks 1 and 2, we have shown that the CaSR transactivates the EGFR (see manuscript in Appendix), thereby completing Task 3. Since the MAP kinase, ERK1/2, is a major signal transduction pathway utilized by the EGFR, we initially documented a delayed phosphorylation of ERK1/2 by Western blotting (Fig. 1A in manuscript in Appendix). Maximal activation was observed at 30 min, and a strong signal persisted at 60 min on Western blots of phospho-ERK1/2. At 120 minutes, in contrast, the signal had nearly disappeared. The phosphorylation of ERK1/2 showed a dependency on the level of  $\text{Ca}^{2+}$  employed; the strongest signal was observed with 7.5 mM  $\text{Ca}^{2+}$ , while signals of intermediate intensity were observed at 1.5 and 3.0 mM  $\text{Ca}^{2+}$  (Fig. 1B).

In order to document that high  $\text{Ca}^{2+}$ -evoked activation of ERK is CaSR-mediated, we examined the effects of a known polycationic CaSR agonist, spermine, and of a selective CaSR activator, NPS R-467, on phospho-ERK1/2. Incubation of PC-3 cells with 100  $\mu\text{M}$  spermine for 30 min increased the level of phospho-ERK1/2 (Fig. 1C). Moreover, NPS R-467 produced a much greater increase in phospho-ERK1/2 than did

its less potent stereoisomer, NPS S-467. Since NPS R-467 is 10 to 100 fold more potent than NPS S-467 in activating the CaSR, our results indicate that high  $\text{Ca}^{2+}$ -induced ERK phosphorylation is mediated by the CaSR.

Next, we examined the effects of various inhibitors and neutralizing antibodies to assess the involvement of transactivation of the EGFR in CaSR-mediated activation of ERK1/2. AG1478, an EGFR kinase inhibitor, and PD98059, a MEK1 inhibitor, inhibited most of the high  $\text{Ca}^{2+}$ -evoked ERK phosphorylation (Fig. 2). GM6001, a pan matrix metalloproteinase (MMP) inhibitor, and antibodies against the EGFR and HB-EGF (heparin-bound EGF) also reduced ERK phosphorylation, consistent with the model of transactivation shown in Fig. 7. In contrast, AG1296, an inhibitor of the platelet-derived growth factor receptor kinase, had no effect on ERK phosphorylation. These results provide indirect evidence that activation of the CaSR transactivates the EGFR, but not the PDGFR, at least in part through activation of MMP(s).

We next directly measured the effect of high  $\text{Ca}^{2+}$  on the extent of phosphorylation of the EGFR. Phosphorylation of the EGFR was assessed using Western analysis with a monoclonal anti-phosphotyrosine antibody following immunoprecipitation of cell lysates with a rabbit polyclonal anti-EGFR antibody. The EGFR was phosphorylated to some extent even under basal (0.5 mM  $\text{Ca}^{2+}$ ) conditions (Fig. 3); following 10 min incubation in medium with 7.5 mM  $\text{Ca}^{2+}$ , however, the phosphorylation of the EGFR increased and was sustained for at least 30 min.

We have previously demonstrated that high  $\text{Ca}^{2+}$  stimulates PTHrP secretion in PC-3 cells (1). This action of  $\text{Ca}^{2+}$  is at least partially mediated by the CaSR, since hormonal secretion is reduced by transfecting the cells with a dominant negative CaSR, and known CaSR agonists, e.g., neomycin and gadolinium, promote PTHrP secretion. Thus, we wondered if the CaSR might stimulate PTHrP secretion through transactivation of the EGFR.

High  $\text{Ca}^{2+}$  dose-dependently stimulated PTHrP secretion in PC-3 cells (Fig. 4). This stimulation was inhibited by 20  $\mu\text{M}$  PD98059 and by 0.7  $\mu\text{M}$  AG1478. In contrast, 1  $\mu\text{M}$  AG1296 had no effect on PTHrP secretion. When the cells were preincubated with anti-HB-EGF antibody for 30 min, 5  $\mu\text{g}/\text{ml}$  of the antibody significantly inhibited PTHrP secretion (by 42%) even under basal conditions (0.5 mM  $\text{Ca}^{2+}$ ) (Fig. 5). At 7.5 mM  $\text{Ca}^{2+}$ , the anti-HB-EGF antibody likewise produced a dose dependent inhibition of PTHrP secretion. The anti-EGFR antibody gave similar results (data not shown). Preincubation with 10  $\mu\text{M}$  GM6001 also reduced PTHrP secretion by 40% at 0.5 mM  $\text{Ca}^{2+}$ , and by about 50% at 3.0 and 7.5 mM  $\text{Ca}^{2+}$  (Fig. 6). These findings indicate that EGF and HB-EGF activate the EGFR even under basal conditions and that high  $\text{Ca}^{2+}$ -induced PTHrP secretion is reduced by blockade of the CaSR-EGFR-ERK pathway. The former result is consistent with the presence of



phosphorylated EGFR at 0.5 mM  $\text{Ca}^{2+}$ , even following serum starvation (Fig. 3).

*Task 4-To show that knocking out the CaSR reduces the severity of bone resorption in the femora of nude mice injected with PC-3 cells (months 6-36).*

We continued during months 12-24 the development of PC-3 cells stably transfected with a dominant negative CaSR or with the corresponding vector. We have transfected PC-3 cells with a standard mammalian expression vector (pcDNA3) and subjected the transfected cells to selection with hygromycin. To date we have not yet been successful in obtaining individual, stably transfected PC-3 clones, in part because the cells grow very slowly at low density. While we have been able to select cells transfected with the dominant negative CaSR that grow in the presence of hygromycin, on immunocytochemistry only about 20% were positive for the CaSR. We plan to develop individual clones of stably transfected with the dominant negative CaSR so as to avoid the apparent heterogeneity in our studies to date.

#### KEY RESEARCH ACCOMPLISHMENTS:

- Shown that high  $\text{Ca}^{2+}$  and EGF stimulate ERK1/2 in PC-3 cells; Furthermore, the polycationic CaSR agonist, spermine, and the potent calcimimetic, NPS R-467, increase ERK1/2 in PC-3 cells to a greater extent than the less potent calcimimetic, NPS R-467, consistent with the mediatory role of the CaSR in this action.
- Demonstrated that an inhibitor of the EGF receptor kinase, a matrix metalloproteinase inhibitor, and antibodies against the EGFR and HB-EGF reduce high  $\text{Ca}^{2+}$ -evoked ERK activation, consistent with the involvement of CaSR-mediated transactivation of the EGFR.
- Shown that high  $\text{Ca}^{2+}$ -stimulated PTHrP secretion is reduced by the EGFR inhibitor, the matrix metalloproteinase inhibitor, and the antibodies to the EGFR and HB-EGF, providing further evidence that the CaSR transactivates the EGFR.
- Documented that high calcium stimulates a time dependent increase in the tyrosine phosphorylation of the EGFR, providing direct evidence for CaSR-mediated transactivation of the CaSR.

#### REPORTABLE OUTCOMES:

During months 12-24, the following manuscript was submitted for publication. The text of the manuscript is at the end of this report.

Yano S, Macleod RJ, Chattopadhyay N, Kifor O, Tfelt-Hansen J, Butters R, and Brown EM. Calcium Sensing Receptor Activation Stimulates Parathyroid Hormone Related Protein Secretion in Prostate Cancer Cells: Role of Epidermal Growth Factor Receptor Transactivation (submitted for publication)

#### CONCLUSIONS:

Our results to date support the major underlying hypotheses driving this research, namely that the CaSR mediates high  $\text{Ca}^{2+}$ -stimulated PTHrP secretion from PC-3 cells and could provide the basis for a "feed-forward" mechanism in vivo that would serve to aggravate the skeletal complications of prostate cancer metastatic to bone. The importance of this research lies in the implication that the CaSR could serve as a therapeutic target for CaSR antagonists that could diminish the severity of the skeletal complications of prostate cancer. Furthermore, it is possible that expression of the CaSR in other cancers that metastasize to bone (e.g., breast cancer) could serve as the mediator of a similar "feed-forward" mechanism and thereby provide the basis for a novel therapy of cancers other than prostate cancer.

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#### APPENDICES:

One manuscript submitted for publication (see following material)



**APPENDIX**

**Calcium Sensing Receptor Activation Stimulates Parathyroid Hormone Related Protein  
Secretion in Prostate Cancer Cells: Role of Epidermal Growth Factor Receptor Transactivation**

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Running title: CaR transactivates EGFR

## Abstract

We have previously reported that high extracellular  $\text{Ca}^{2+}$  stimulates parathyroid hormone related protein (PTHrP) release from human prostate and breast cancer cell lines as well as from H-500 rat leydig cancer cells, an action mediated by the calcium sensing receptor (CaR). Activating the CaR leads to phosphorylation of mitogen-activated protein kinases (MAPKs) that participate in PTHrP synthesis and secretion. Since the CaR is a G protein-coupled receptor, it is likely to transactivate the epidermal growth factor receptor (EGFR) or the platelet-derived growth factor receptor (PDGFR). In this study, we hypothesized that activation of the CaR transactivates the EGFR and/or PDGFR, and examined whether transactivation affects PTHrP secretion in PC3 human prostate cancer cells. Using Western analysis, we observed that an increase in extracellular  $\text{Ca}^{2+}$  resulted in delayed activation of extracellular signal-regulated kinase (ERK) in PC3 cells. Preincubation with AG1478 (an EGFR kinase inhibitor) or an EGFR neutralizing antibody inhibited the high  $\text{Ca}^{2+}$ -induced phosphorylation of ERK1/2. GM6001, a pan matrix metalloprotease (MMP) inhibitor, also partially suppressed the ERK activation, but AG1296 (a PDGFR kinase inhibitor) did not. High extracellular  $\text{Ca}^{2+}$  stimulates PTHrP release during a 6 hour incubation (1.5-2.5 and 3-4 fold increases in 3.0 and 7.5 mM  $\text{Ca}^{2+}$ , respectively). When cells were preincubated with AG1478, GM6001, or an antihuman heparin bound EGF (HB-EGF) antibody, PTHrP secretion was significantly inhibited under basal as well as high  $\text{Ca}^{2+}$  conditions, while AG1296 had no effect on PTHrP secretion. Taken together, these findings indicate that activation of the CaR transactivates the EGFR, but not the PDGFR, leading to phosphorylation of ERK1/2 and resultant PTHrP secretion. This transactivation is most likely mediated by activation of MMP and cleavage of proHB-EGF to HB-EGF.

## Introduction

Prostate cancer is known as the second most deadly cancer in men in the United States [1]. In most cases, prostate cancer metastasizes to bone, which negatively impacts prognosis [2]. Previous studies demonstrated expression of parathyroid hormone-related protein (PTHrP) in normal and malignant prostate epithelial cells [3, 4]. PTHrP, which was originally isolated from renal, lung, and breast cancers in 1987, plays an important role in normal bone formation, development of mammary gland, skin, and teeth, and regulation of the contractility of smooth muscle [5]. Since the aminoterminal of PTHrP has structural similarity to PTH, they can act on the same receptor, the type 1 PTH receptor (PTH1R). However, PTHrP acts on cells in a paracrine, autocrine and/or intracrine fashion, whereas PTH acts in an endocrine manner [6]. In the prostate gland, the physiological role of PTHrP is unknown. However, evidence that there is 1) higher PTHrP expression in intraepithelial neoplasia than in normal prostate epithelium and 2) higher PTHrP expression in prostate carcinoma than in benign hyperplasia, suggest that there are promalignant or proliferative effects of PTHrP that participate in the pathophysiology of prostate cancer [6-8].

We have previously reported that high concentrations of extracellular calcium ( $\text{Ca}^{2+}_o$ ) stimulate PTHrP secretion from rat H-500 leydig cells, human embryonic kidney cells stably transfected with the calcium-sensing receptor (CaR), human breast cancer cell lines, prostate cancer cell lines, and human astrocytes, astrocytomas and meningiomas, and that this phenomenon is mediated by the CaR expressed on these cells [9-13]. These findings suggest the existence of a vicious cycle that could contribute to the pathophysiology of humoral hypercalcemia of malignancy (HHM) and osteolytic bone metastases. Once PTHrP-producing cancer cells metastasize to bone, for example, locally high

levels of  $\text{Ca}^{2+}_o$  could stimulate PTHrP secretion further. Excessive production of PTHrP, in turn, would elevate local and/or systemic levels of  $\text{Ca}^{2+}_o$  through the PTH1R expressed on renal tubules and osteoblasts.

The CaR that was first cloned from bovine parathyroid gland has a central role in the regulation of PTH secretion and calcium metabolism [14, 15]. Although the CaR is expressed mainly on the parathyroid glands, distal tubules of the kidney and the thyroid C cells, the receptor has been identified in intestinal epithelial cells, bone cells, several nephron segments other than the distal tubule, and many other tissues and cell lines [16]. The CaR activates MAP kinases (ERK, p38 MAPK, JNK/SAPK) in certain cells, which may mediate some of the known biological actions of the CaR [17-21]. In previous reports, we have demonstrated that MAP kinase pathways play key roles in CaR-stimulated PTHrP secretion [10, 21]. However, it remains unclear how the CaR activates MAP kinases. Since the CaR is a member of the superfamily of G protein-coupled receptors (GPCR), we hypothesized that the CaR activates receptor tyrosine kinases (RTKs), such as the epidermal growth factor receptor (EGFR) or platelet-derived growth factor receptor (PDGFR) and, in turn, MAPKs. Recent evidence suggests that transactivation of the EGFR by GPCRs is mediated by activation of one or more metalloproteinases, which cleave proheparin binding EGF (proHB-EGF) to release HB-EGF [22, 23]. This mechanism of GPCR-induced EGFR activation, which has been called the 'triple-membrane-passing-signaling' model, has been widely accepted [24]. Thus, we wondered if the CaR could also transactivate the EGFR. In this study, we show that the CaR transactivates the EGFR at least in part via metalloproteinase activation, followed by ERK phosphorylation, and that CaR-induced EGFR transactivation stimulates PTHrP secretion in PC3 human prostate cancer cells.

## Materials and Methods

### Materials:

Selective inhibitors of MEK1 (PD98059), EGFR kinase (AG1478), PDGFR kinase (AG1296), and pan MMPs (GM6001) were all obtained from Calbiochem-Novabiochem (San Diego, CA). Neutralizing antibodies against the EGFR and HB-EGF were obtained from R&D Systems (Minneapolis, MN). Polyclonal antisera to the EGFR and a mouse monoclonal antibody against phosphotyrosine, PY99, were purchased from Santa Cruz (Santa Cruz, CA). A polyclonal antiserum to phosphorylated ERK1/2 and a mouse monoclonal antibody against ERK2 were purchased from New England Biolabs (Beverly, MA). The enhanced chemiluminescence kit, Supersignal, was purchased from Pierce (Rockford, IL). Protease inhibitors were from Boehringer Ingelheim, and all other reagents were from Sigma Chemical Co. (St. Louis, MO).

### Cell Culture:

The PC-3 human prostate cancer cell line was obtained from the American Type Culture Collection (Rockville, MD). The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin-100 µg/ml streptomycin and grown at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. Cells were passaged every 4 to 5 days using 0.05% trypsin-0.53 mM EDTA. FBS was obtained from Gemini Bio-Products (Calabasas, CA), and other cell culture reagents were purchased from GIBCO-BRL (Grand Island, NY).

### PTHrP secretion studies:

PTHrP secretion from PC-3 cells was determined using the same system as previously described [12]. Briefly, for studies on the effects of the CaR agonist,  $\text{Ca}^{2+}_o$ , and various inhibitors on PTHrP secretion, cells were seeded in 96-well plates ( $5 \times 10^3$  cells/well) in 0.15 ml of growth medium. After 72 h, the growth medium was removed and replaced with 0.15 ml of  $\text{Ca}^{2+}$ -free DMEM containing 4 mM L-glutamine, 0.2% BSA, 100 U/ml penicillin-100  $\mu\text{g/ml}$  streptomycin, and 0.5 mM  $\text{CaCl}_2$ . Two hours later, this medium was removed and replaced with 0.275 ml of the same medium or that supplemented with additional  $\text{CaCl}_2$  (to a final concentration of 1.5, 3.0, or 7.5 mM) for 6 hours. All inhibitors and neutralizing antibodies were preincubated 30 min before replacement with the test medium. Six hours later, the conditioned medium was collected to measure PTHrP content. Each experiment was carried out at least three times, and duplicate incubations were performed for each treatment.

PTHrP was measured in conditioned medium using a two-site immunoradiometric assay (Nichols Institute Diagnostics, San Juan Capistrano, CA) that detects PTHrP-(1-72) with a sensitivity of  $\sim 0.3$  pmol/L. PTHrP assays were initiated immediately after collection of the conditioned medium to minimize degradation of the peptide resulting from freeze-thawing and other manipulations. Standard curves of PTHrP concentrations were generated with the addition of recombinant PTHrP-(1-86) to the treatment medium used in this study (i.e., unconditioned  $\text{Ca}^{2+}$ -free DMEM containing 0.5 mM  $\text{CaCl}_2$ ). Calcium and other reagents alone had no effect on the PTHrP assay when added in the absence of PC-3 cell-conditioned medium.

Western blot analysis:



For the determination of ERK1/2 phosphorylation, monolayers of PC-3 cells were grown in six-well plates. When cells reached 50% confluency, they were incubated for 48 h in serum-free,  $\text{Ca}^{2+}$ -free DMEM containing 4 mM L-glutamine, 0.2% BSA, and 0.5 mM  $\text{CaCl}_2$ . This medium was then removed and replaced with the same medium supplemented with either 7.5 mM  $\text{CaCl}_2$  alone or 30 ng/ml EGF. All inhibitors and neutralizing antibodies were preincubated 30 min before replacement of test medium. At the end of the incubation period, the medium was removed, the cells were washed twice with ice-cold phosphate-buffered saline (PBS) containing 1 mM sodium vanadate and 25 mM NaF, and then 100  $\mu\text{L}$  of ice-cold lysis buffer was added [20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 25 mM NaF, 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol, 1 mM sodium vanadate, 50 mM glycerophosphate, and a cocktail of protease inhibitors]. These were aprotinin, leupeptin, soybean trypsin inhibitor, pepstatin, and calpain inhibitor (10  $\mu\text{g}/\text{ml}$  of each), as well as 100  $\mu\text{g}/\text{ml}$  of Pefabloc; all were added from frozen stocks, except the sodium vanadate, which was freshly prepared on the day of the experiment. The cells were scraped into the lysis buffer, sonicated for 10 sec, and then centrifuged at  $6,000 \times g$  for 5 min at  $4^\circ\text{C}$ . The supernatants were kept at  $-20^\circ\text{C}$ . These protein samples were saved for Western blotting as previously described [21]. Briefly, equal amounts of supernatant protein were separated by SDS-PAGE. The separated proteins were electrophoretically transferred to nitrocellulose membranes (Schleicher and Schuell) and incubated with blocking solution (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, and 0.25% BSA) containing 5% dry milk for at least 1 h at room temperature. ERK1/2 phosphorylation was detected by immunoblotting using an 18 h incubation with a 1:1000 dilution of a rabbit polyclonal antiserum specific for phospho-ERK1/2. Blots were washed for five 15-min periods at room temperature (1% PBS, 1% Triton X-100, and 0.3 % dry milk) and then incubated for 1 h with a second, goat anti-rabbit, peroxidase-linked antiserum (1:2000) in blocking solution. After washing the membrane, bands were

visualized by chemiluminescence according to the manufacturer's protocol (Supersignal, Pierce Chemical). The same membrane was used after stripping (Restore Western Blot Stripping, Pierce) for determination of total ERK2 to confirm equal loading of all the lanes. Protein concentrations were measured with the Micro BCA protein kit (Pierce).

#### Immunoprecipitation:

After serum starvation for at least 48 hours, cells were stimulated with 7.5 mM  $\text{Ca}^{2+}_o$  as described above. At the indicated time points, cells were washed with ice-cold PBS and lysed with immunoprecipitation buffer containing 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.2 mM sodium vanadate, and protease inhibitors (as described above). The cell lysate was centrifuged at 10,000 x g for 10 min. For immunoprecipitation, equal amounts of protein were incubated with polyclonal EGFR antibody overnight, and then incubated with protein A/G agarose beads for a further 1 hour at 4°C. Bound immune complexes were washed three times with immunoprecipitation buffer containing protease and phosphatase inhibitors and detergents. The pellet was eluted by boiling for 5 min with 2 x Laemmli sample buffer. Supernatant proteins were separated by 7.0 % SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with monoclonal antiphosphotyrosine antibody, PY99. The stripped membrane was then reblotted with EGFR antibody.

#### Statistics:

The data are presented as the mean  $\pm$  SE of the indicated number of experiments. Data were analyzed using one-way ANOVA or students *t*-test. A P value of <0.05 was considered to indicate a statistically significant difference.

## Results

We have previously reported that ERK activation plays a critical role in high  $\text{Ca}^{2+}_o$ -induced PTHrP secretion, and that high  $\text{Ca}^{2+}_o$  produces a delayed phosphorylation of ERK1/2 in the cell types studied to date [10, 21]. In PC3 cells, we also confirmed a delayed phosphorylation of ERK1/2 by Western blotting (Fig. 1A). Maximal activation was present at 30 min, and a strong signal was still present on Western blots of phospho-ERK1/2 at 60 min. At 120 minutes, in contrast, the signal had almost disappeared. The magnitude of the phosphorylation of ERK1/2 is dependent on the  $\text{Ca}^{2+}_o$  concentration employed, as the strongest signal was observed with 7.5 mM  $\text{Ca}^{2+}_o$ , while intermediate signals were observed with 1.5 and 3.0 mM  $\text{Ca}^{2+}_o$  (Fig. 1B).

### CaR activates ERK

In order to make sure that the ERK activation is mediated by the CaR, we examined the effects of a known CaR agonist, spermine and of a selective CaR activator, NPS R-467 [25]. When cells were incubated with 100  $\mu\text{M}$  spermine for 30 min, the signal was increased over basal activity (Fig. 1C). In addition, a much stronger signal was observed in cells incubated with NPS R-467 than in those exposed to the less potent stereoisomer, NPS S-467. Since NPS R-467 is 10 to 100 fold more potent than NPS S-467 [25], our results indicate that high  $\text{Ca}^{2+}_o$ -induced ERK phosphorylation is mediated by the CaR.

### Evidence for EGFR transactivation

Next, we examined the effects of various inhibitors and neutralizing antibodies as described below. In Fig. 2, AG1478, which is an EGFR kinase inhibitor, and PD98059, which is a MEK1 inhibitor,

inhibited most of the high  $\text{Ca}^{2+}_o$ -induced ERK phosphorylation. GM6001, a pan matrix metalloprotease (MMP) inhibitor, and antibodies against the EGFR as well as HB-EGF also diminished ERK phosphorylation. However, AG1296, a PDGFR kinase inhibitor, did not affect the level of ERK phosphorylation. These findings suggest that activation of the CaR results in transactivation of the EGFR, but not of the PDGFR, at least in part through activation of MMP(s).

Finally, we examined the extent of phosphorylation of the EGFR using immunoprecipitation. Phosphorylation of the EGFR was assessed using Western analysis with a monoclonal anti-phosphotyrosine antibody following immunoprecipitation of cell lysates with a rabbit polyclonal anti-EGFR antibody. Fig. 3 shows that the EGFR was phosphorylated to some extent even under basal (0.5 mM  $\text{Ca}^{2+}_o$ ) conditions; after 10 min incubation in medium with 7.5 mM  $\text{Ca}^{2+}_o$ , however, the phosphorylation of the receptor increased and was sustained for at least 30 min.

#### Effect of EGFR and PDGFR inhibitors on PTHrP secretion

We have previously demonstrated that high  $\text{Ca}^{2+}_o$  stimulates PTHrP secretion in PC3 cells [12]. This action of  $\text{Ca}^{2+}_o$  is at least partly mediated by the CaR, since hormone secretion is attenuated after transfection of the cells with a dominant negative CaR, and known CaR agonists, e.g., neomycin and gadolinium, promote PTHrP secretion [12]. Thus, we wondered if the CaR might stimulate PTHrP secretion through transactivation of the EGFR.

High  $\text{Ca}^{2+}_o$  stimulated PTHrP secretion in PC3 cells in a dose dependent manner (Fig.4). This stimulation was inhibited by 20  $\mu\text{M}$  PD98059 and by 0.7  $\mu\text{M}$  AG1478. However, 1  $\mu\text{M}$  AG1296 did not affect PTHrP secretion. When the cells were preincubated with anti-HB-EGF antibody for 30 min,

5  $\mu\text{g/ml}$  of the antibody significantly inhibited PTHrP secretion (by 42 %) even under basal conditions (0.5 mM  $\text{Ca}^{2+}_o$ ) (Fig. 5). At 7.5 mM  $\text{Ca}^{2+}_o$ , the anti-HB-EGF antibody suppressed PTHrP secretion in a dose dependent fashion. The anti-EGFR antibody gave similar results (data not shown).

Preincubation with 10  $\mu\text{M}$  GM6001 also suppressed PTHrP secretion by 40 % at 0.5 mM  $\text{Ca}^{2+}_o$ , and by about 50 % in 3.0 and 7.5 mM  $\text{Ca}^{2+}_o$  medium (Fig. 6). These findings indicate that EGF or HB-EGF activates the EGFR even under basal conditions and that high  $\text{Ca}^{2+}_o$ -induced PTHrP secretion is suppressed by blockade of the CaR-EGFR-ERK pathway. The former result seems compatible with the presence of phosphorylated EGFR at 0.5 mM  $\text{Ca}^{2+}_o$  even after serum starvation.

## Discussion

EGF is a known inducer of PTHrP secretion in human prostate tissue, mammary epithelial cells, bone, breast, kidney and lung cell lines, keratinocytes, osteosarcoma cells, epithelial cancer cells, and rat leydig tumor cells [26-32]. This induction has been reported to involve both transcriptional and posttranscriptional mechanisms [32-34]. The EGF-induced increase in PTHrP synthesis is most likely mediated by tyrosine kinase but not by cAMP in cultured mammary epithelial cells [29], where an additive effect of PMA and EGF on the induction of PTHrP transcription was shown, indicating that PKC activation could also be involved in this stimulation. We confirmed that EGF significantly stimulated ERK activation at 3 min and PTHrP secretion at 6 hours in PC3 cells (data not shown).

PTHrP participates in promoting the growth in PC-3 cells, which express a functional PTH1R [6]. Previous work has shown that the level of PTHrP expression is higher in prostate cancer than in normal prostate tissue and that PC-3 cells secrete a significantly higher amount of PTHrP-1-34 than do the DU-145 and LNCaP prostate cancer cell lines [35, 36]. PTHrP and the PTH1R are coexpressed in both primary prostate cancers as well as in bone metastases [37]. In addition, PTHrP seems to influence cell adhesion by enhancing the synthesis of several extracellular matrix proteins and some integrin subunits [38]. These findings suggest that PTHrP may also play a critical role in promoting tumor invasiveness and skeletal metastases through paracrine/autocrine and probably intracrine mechanisms [6], although overexpression of PTHrP did not accelerate bone metastasis in a murine breast cancer model [39]. In an in vivo study, neutralizing antibodies to PTHrP or guanine-nucleotide analogs, which inhibit PTHrP gene transcription, not only decreased osteoclastic bone resorption but



also inhibited the development of metastases to bone by human breast cancer cells [40, 41].

Furthermore, the intracrine actions of PTHrP can prevent apoptosis under certain circumstances [42].

In the present study, we showed that high  $\text{Ca}^{2+}_o$  stimulates PTHrP secretion via CaR-mediated activation of ERK in PC3 cells and demonstrated that this activation of ERK is mediated by transactivation of the EGFR through activation of MMPs, followed by cleavage of proHB-EGF to HB-EGF. The CaR belongs to the superfamily of GPCRs, some of which have previously been shown to be associated with receptor tyrosine kinases (RTKs); for example, the angiotensin II AT1 receptor, bradykinin B2 receptor, vasopressin V1 receptor, cholecystokinin CCK1R, gastrin CCK2R, and bombesin receptors stimulate MAPK cascades via activation of  $G_{q/11}$  followed by transactivation of the EGFR [22, 43-49]. Since these peptide receptors can also activate  $G_i$  proteins, there appear to be two pathways activating the MAPK cascade via  $G_i$ . Activation of  $G_{q/11}$  and  $G_i$  can stimulate PKC and PI3K, leading to Raf-MEK-ERK activation with convergence of the signaling cascade. Since the CaR is thought to couple to both  $G_{q/11}$  and some isoforms of  $G_i$  [14-17], it could activate the dual pathways, PKC and PI3K, as well as transactivate the EGFR. In fact, preincubation with either PKC or PI3K inhibitors suppressed ERK activation and PTHrP secretion to some extent in PC3 cells (Yano et al., unpublished data).

Angiotensin II-induced activation of MAPK is partly mediated by transactivation of the PDGFR in human mesangial cells [50]. In the present study, however, the CaR does not appear to transactivate the PDGFR in PC3 cells. This difference between the CaR and the angiotensin II receptor could be receptor- or cell type-dependent. For example, it seems to be unlikely that the CaR can transactivate EGFR in the parathyroid gland in vivo. Nevertheless, further studies are needed to clarify the

downstream of the CaR signaling and pathological/physiological relevance of transactivation.

Although caveolin, which is one of the components of caveolae, and filamin, a scaffold protein, would play a critical role in the CaR signaling, little is known about contribution to RTKs activation [51, 52].

Transactivation of RTKs has been considered as a mitogenic pathway for GPCRs. Since high  $\text{Ca}^{2+}_o$  stimulates cell proliferation via the CaR in several cell types [53-57], the CaR-EGFR-ERK pathway could be involved in such cases, although other pathways, i.e., p38 MAPK or PI3K, might also participate in regulating proliferation [58, 59]. When we evaluated cell proliferation 6 hours after stimulation with high  $\text{Ca}^{2+}_o$ , there was no significant change, suggesting that CaR-induced changes in cell number did not affect the present data. Yet, since high  $\text{Ca}^{2+}_o$  most probably stimulates cell proliferation as well as PTHrP secretion via EGFR transactivation in PTHrP-producing cancer cells, further study is necessary to evaluate any effects of the CaR on cell proliferation and survival.

In conclusion, activation of the CaR transactivates the EGFR, but not the PDGFR, leading to activation of ERK1/2 and resultant PTHrP secretion in PC-3 cells. This transactivation is most likely mediated by activation of MMP and subsequent cleavage of proHB-EGF to HB-EGF.

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## Figure Legends

Fig. 1: High  $\text{Ca}^{2+}_o$  activates ERK via the CaR in PC3 cells.

Cells were serum starved overnight in test medium ( $0.5 \text{ mM Ca}^{2+}_o$ ), and were stimulated by medium with  $7.5 \text{ mM Ca}^{2+}_o$ . Protein was collected at each time point for Western blotting (Fig. 1A).

Incubation with  $30 \text{ ng/ml EGF}$  for  $10 \text{ min}$  served as a positive control. Equal amounts of total cellular protein were separated by electrophoresis with  $12 \%$  polyacrylamide gels, transferred to nitrocellulose membranes, and analyzed by immunoblotting using a polyclonal antibody against phosphorylated ERK1/2 and a monoclonal antibody against ERK2 protein. Cells were also stimulated with different concentration of  $\text{Ca}^{2+}_o$  (Fig. 1B) and the CaR agonists (Fig. 1C) for  $30 \text{ min}$ . Experiments were repeated three times, and the data from a representative one are shown.

Fig. 2: High  $\text{Ca}^{2+}_o$ -induced ERK phosphorylation is mediated by EGFR activation.

Cells were serum starved overnight, and preincubated with the inhibitors or antibodies indicated. The cells were then treated with  $0.5$  and  $7.5 \text{ mM Ca}^{2+}_o$  in lane 1 and 2-8, respectively, for  $30 \text{ min}$ . Equal amounts of total cellular protein were separated by electrophoresis on  $12 \%$  polyacrylamide gels, transferred to nitrocellulose membranes, and analyzed by immunoblotting using a polyclonal antibody against phosphorylated ERK1/2 and a monoclonal antibody against ERK2 protein. Experiments were repeated three times, and the data shown are similar to those from the other two experiments.

Fig. 3: High  $\text{Ca}^{2+}_o$  induces EGFR phosphorylation in PC3 cells.

Cells were serum starved for  $48 \text{ hours}$  in test medium ( $0.5 \text{ mM Ca}^{2+}_o$ ) and were stimulated by medium with  $7.5 \text{ mM Ca}^{2+}_o$ . Cells were lysed to collect protein at each time point. Total EGFR protein was

immunoprecipitated with a polyclonal EGFR antibody and subsequent addition of protein A/G agarose. The protein samples were separated by electrophoresis on 7.0 % polyacrylamide gels, transferred to nitrocellulose membranes, and immunoblotted with a monoclonal antibody against phosphotyrosine, PY99. The membrane was stripped and reblotted with a polyclonal antibody against the EGFR. For more details, see Material and Methods.

Fig. 4: Effects of inhibitors of the phosphorylation of MEK1, EGFR and PDGFR on high  $\text{Ca}^{2+}_o$ -induced PTHrP secretion in PC3 cells.

Cells were washed once with test medium (0.5 mM  $\text{Ca}^{2+}_o$ ) and incubated for 6 hours in test medium with various  $\text{Ca}^{2+}_o$  concentrations after preincubation with or without inhibitors for 30 min. The conditioned medium was collected to determine the amount of PTHrP released during the incubation. PTHrP secretion was measured by IRMA as described in Materials and Methods, and was expressed as fold increase (percent) relative to control. N=3, mean $\pm$ -SEM; \*p < 0.05 vs. 0.5 mM  $\text{Ca}^{2+}_o$ , #p < 0.05 vs. no inhibitor group in corresponding  $\text{Ca}^{2+}_o$ .

Fig. 5: Neutralizing antibody against HB-EGF inhibits basal and high  $\text{Ca}^{2+}_o$ -induced PTHrP secretion in PC3 cells.

Cells were preincubated with various concentrations of HB-EGF antibody for 30 min, and treated with low or high  $\text{Ca}^{2+}_o$  for 6 hours. PTHrP released into the medium was determined by IRMA as described in Materials and Methods was expressed as fold increase (percent) relative to control. N=3, mean $\pm$ -SEM; \*p < 0.05 vs. no HB-EGF antibody group in corresponding  $\text{Ca}^{2+}_o$ .

Fig. 6: MMP inhibitor suppresses PTHrP secretion in low as well as high  $\text{Ca}^{2+}_o$ .

Cells were preincubated with 10  $\mu$ M GM6001 for 30 min, and were treated with low and high  $\text{Ca}^{2+}_o$  for 6 hours. PTHrP released into the medium was determined as described in Materials and Methods and expressed as fold increase (percent) relative to control. N=3, mean $\pm$ -SEM; \*p < 0.05 vs. corresponding basal.

Figure 7: Schematic delineation of the mechanism by which the CaSR transactivates the EGFR.

The CaSR, presumably acting via the G protein,  $G_{q/11}$ , activates a matrix metalloprotease(s) by an unknown mechanism, which then cleaves proHB-EGF to soluble HB-EGF. The latter then activates the EGFR, which activates ERK1/2 via the Ras, Raf, MEK1/2 pathway and, as a consequence, stimulates PTHrP production.

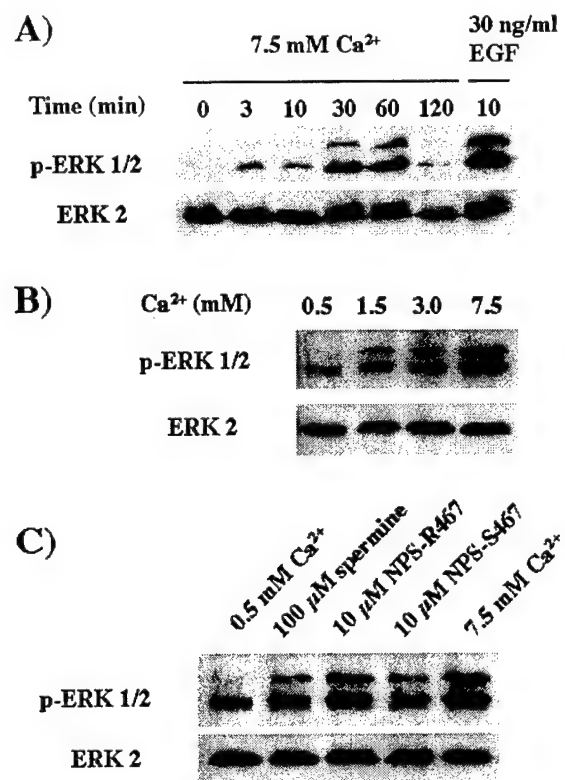


Figure 1

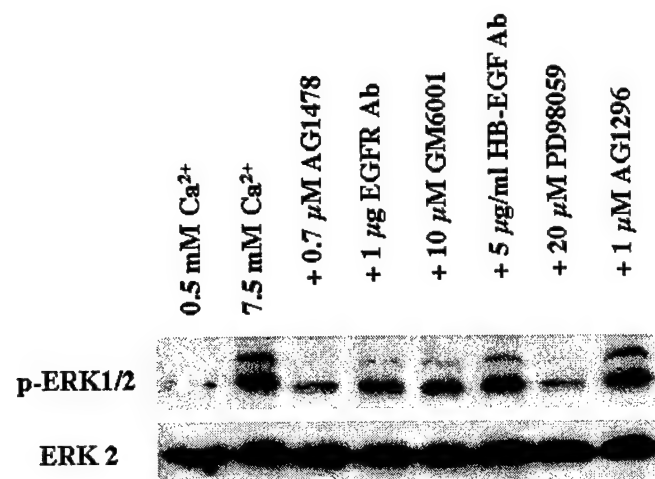


Figure 2

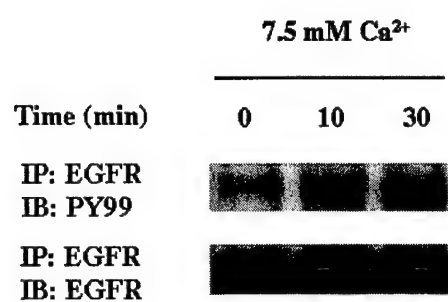


Figure 3

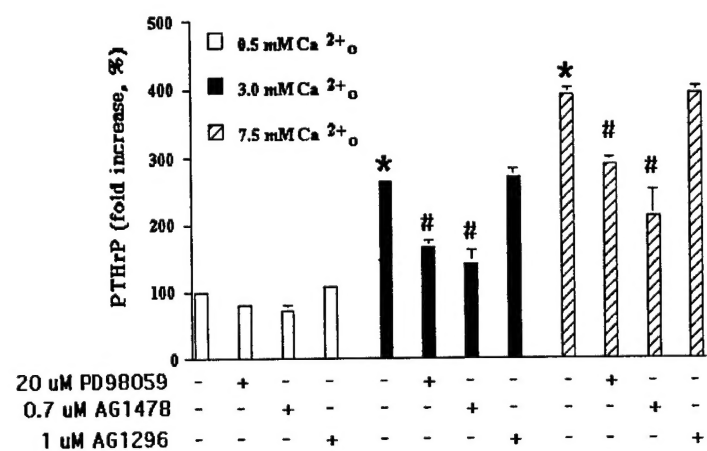


Figure 4

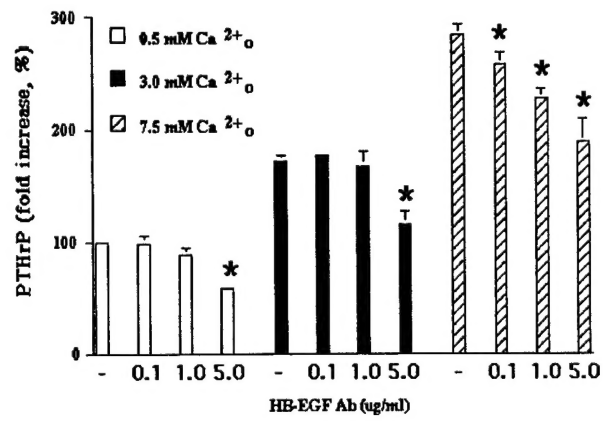


Figure 5



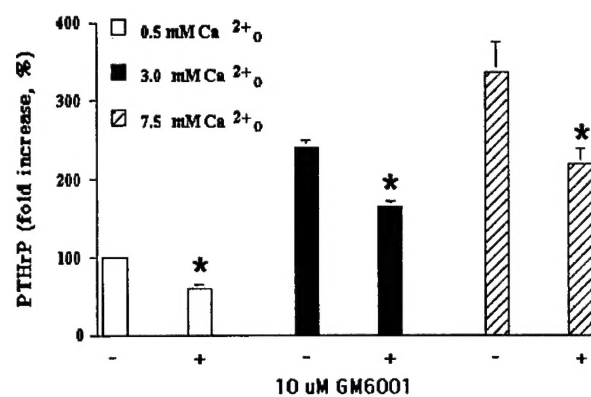


Figure 6

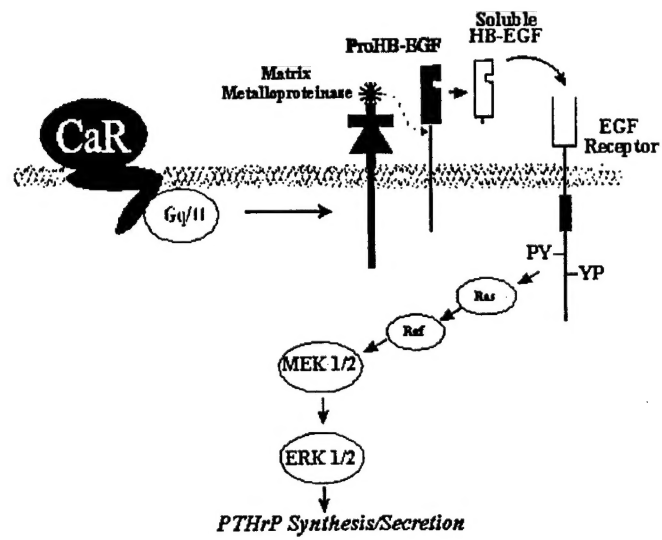


Figure 7